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(54) Title: A NOVEL FORM OF DIPEPTIDYLPEPTIDASE IV (CD26) FOUND IN HUMAN SERUM, ANTIBODIES THERETO, AND USES THEREFOR (57) Abstract A circulating, soluble form of DPPIV/CD26 isolated from human serum is disclosed. The serum form shares similar enzymatic and antigenic properties with the ubiquitous membrane form. However, in several biochemical aspects there are distinct differences. In particular, the circulating serum form has a molecular weight of 175 kDa (in contrast to the 105 kDa molecular weight of the membrane form), and it does not bind Adenosine Deaminase Type-1. Nevertheless, the circulating form expresses functional dipeptidylpeptidase IV activity and retains the ability to costimulate the T lymphocyte response to recall antigen. Circulating DPPIV has been determined to be the soluble form of a 175 kDa DPPIVCD26-related molecule rapidly expressed on the surface of activated T cells, prior to the expression of 105 kDa CD26. Although 105 kDa membrane type CD26 may be found in the serum in small amounts, the majority of serum DPPIV activity is provided by a novel peptidase structurally distinct from 105 kDa CD26/DPPIV. Polyclonal and monoclonal antibodies capable of distinguishing the 175 kDa form from the 105 kDa form are also disclosed.		

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A NOVEL FORM OF DIPEPTIDYLPEPTIDASE IV (CD26) FOUND
IN HUMAN SERUM, ANTIBODIES THERETO, AND USES THEREFOR

RELATED APPLICATION

This application is a continuation-in-part of U.S.
Patent Application Ser. No. 08/457,694, filed June 1, 1995,
the whole of which is hereby incorporated by reference
5 herein.

FIELD OF THE INVENTION

This invention relates to human T cell activation
antigens.

GOVERNMENT RIGHTS

10 This invention was made at least in part with funds
provided under grants from the United States Government
(National Institutes of Health Grants AI23360-08, AI12069,
CA55601 and CA34183). Therefore, the U.S. Government has
certain rights in this invention.

15 BACKGROUND OF THE INVENTION

Human CD26, a Type II membrane glycoprotein with
intrinsic dipeptidylpeptidase IV (DPPIV) activity and ability
to bind Adenosine Deaminase Type I (ADA-1), is expressed on
epithelial cells constitutively, but on T lymphocytes its
20 expression is regulated.

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Initially identified as a 105 kDa T cell activation antigen defined by the monoclonal antibody Ta1 (1), the CD26 antigen was subsequently shown to delineate the T cell subset responding to recall antigens (2, 3). Although the antigen is expressed in the liver, kidney and intestine (4), only in the T cell are the levels of membrane CD26 under tight cellular regulation with expression upregulated upon cell activation. CD26 has been shown to have dipeptidylpeptidase IV activity (DPPIV, EC 3.4.14.5) in its extracellular domain (5, 6) and the costimulatory potential appears to be partially dependent upon this enzyme activity (7) which can cleave amino terminal dipeptides with proline, and less effectively, alanine in the penultimate position (8). Although a substrate of relevance to T cell activation has not yet been identified, other substrates, including the neuropeptide substance P, may be processed in vivo by DPPIV/CD26 (9).

CD26 not only marks the activated state but is itself involved in the signal transducing process: crosslinking of CD3 and CD26 results in enhanced T cell activation in the absence of antigen-presenting cells (10). It is unlikely that CD26 is directly involved in transducing the activation signal across the T cell membrane since it has only a very short cytoplasmic region of 6 amino acids (11). The protein tyrosine phosphatase, CD45RO, has been shown to associate with CD26 and may provide a putative mechanism for the costimulation (12). Other associations include the strong binding of Adenosine Deaminase Type I (ADA-1) to CD26 (13).

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This may be of particular importance since ADA activity helps regulate the early stages of signal transduction in T lymphocytes (14). That the costimulatory potential of CD26 occurs extracellularly has been confirmed by showing that a soluble recombinant CD26 (rsCD26) representing the extracellular domain can enhance the T cell-mediated reaction to recall antigens (15). Reinforcing the proposal that soluble CD26 is costimulating, it has been found that in the absence of recall antigen, the rsCD26 has no effect upon the proliferative response. A natural form of soluble DPPIV/CD26 can be identified in normal human serum. The levels of this naturally-occurring soluble DPPIV influenced the level of reactivity of T cells to recall antigens (15).

SUMMARY OF THE INVENTION

We have now isolated soluble DPPIV/CD26 from human serum and have unexpectedly determined that while the serum form shares similar enzymatic and antigenic properties with the membrane form, in several biochemical aspects there are distinct differences. In particular, the soluble form has a molecular weight of 175 kDa, and it does not bind ADA-1. Nevertheless, it retains the ability to costimulate the T lymphocyte response to the recall antigen, tetanus toxoid. Furthermore, N-terminal sequencing of the resulting peptides after tryptic digestion suggested structural disparity between membrane CD26 and soluble serum DPPIV. Accordingly, we suggest that although 105 kDa membrane type CD26 may be found in the serum in small amounts, the majority of serum

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DPPIV activity is provided by a novel peptidase structurally distinct from DPPIV/CD26.

Thus, in one aspect the invention features a substantially purified glycoprotein, a monomer of the glycoprotein having a molecular weight of approximately 175 kDa, of which approximately 130 kDa is the unglycosylated moiety. The glycoprotein of the invention has an antigenic determinant or determinants immunologically cross-reactive with determinants of CD26, is capable of expressing functional dipeptidylpeptidase IV (DPPIV) activity, but is not capable of binding Adenosine Deaminase Type-1 (ADA-1). Preferably, the glycoprotein of the invention exists in the form of a trimer and contains N-linked glycosylation but not O-linked glycosylation.

By "substantially pure" is meant a polypeptide or protein which has been separated from biological macromolecules (e.g., other proteins, carbohydrates, etc.) with which it naturally occurs. Typically, a protein or polypeptide of interest is substantially pure when less than 25% (preferably less than 15%) of the dry weight of the sample consists of such other molecules.

The invention furthermore features the unglycosylated moiety of the above glycoprotein as a substantially purified protein having a molecular weight of approximately 130 kDa.

Also featured is a therapeutic composition containing the DPPIV/CD26 glycoprotein of the invention in a pharmaceutically acceptable carrier (e.g., saline or any aqueous or nonaqueous substance which is suitable for

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injection). Such a therapeutic composition can be used in a method for modulating the immune response of a patient (e.g., enhancing the immune response of an immunosuppressed patient) by administering the composition to the patient by an appropriate means. It is expected to be particularly useful for the treatment of immunosuppression in a patient infected with human immunodeficiency virus (HIV) and having the acquired immune deficiency syndrome (AIDS) or AIDS-related complex, but may also be used where the patient's immune system is depressed as a result of treatment with an immunosuppressive compound, or acquired immunodeficiency of undetermined etiology, or congenital immunodeficiency, such as autoimmune diseases. The soluble, naturally occurring high molecular weight form of DPPIV/CD26 disclosed herein will be particularly useful because the type and extent of glycosylation is the naturally occurring pattern. Therefore, the high molecular weight form should have a longer half life than any soluble recombinant form of CD26.

The compounds of the invention, when combined with a pharmaceutically acceptable carrier, are also useful as vaccine adjuvants, to be administered to an individual vaccinee in conjunction with (i.e., immediately before, after, or along with) a vaccine antigen in order to enhance the immune response produced by such antigen. Examples of vaccine antigens which may be used with the adjuvant of the invention include those containing chemically inactivated or genetically engineered viral or bacterial products, such as

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diphtheria or pertussin toxoid or recombinant viral proteins, and those containing live but attenuated virus or bacteria.

Fragments of serum soluble DPPIV/CD26 can be assayed for costimulatory activity. One such assay would include the following steps: (a) contacting a lymphocyte with a candidate fragment of serum soluble DPPIV/CD26, and (b) determining whether the fragment increases the rate of proliferation of the lymphocyte in response to antigenic stimuli, such increase being an indication of costimulatory activity.

The invention also features nucleic acid encoding 175 kDa DPPIV/CD26, which is useful for transferring expression of the 175 kDa form, either in vivo or in vitro, to cells lacking such capability.

In another aspect the invention features antibodies, polyclonal or monoclonal, and immunoreactive fragments and derivatives thereof, that bind specifically to the 175 kDa form but not to the 105 kDa form of DPPIV/CD26. An individual such antibody recognizes a unique epitope on the 175 kDa form of DPPIV. As used herein, the phrase "unique epitope" refers to any epitope on the 175 kDa form of DPPIV/CD26 that is not found on the 105 kDa form. The antibodies, fragments and derivatives of the invention are capable of differentiating these two forms of DPPIV/CD26 and are, therefore, useful reagents for identifying, quantifying and/or purifying activated T lymphocytes and also for diagnosis or for monitoring the course of various immune diseases or disorders. For example, an antibody of the

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invention can serve as a diagnostic agent in a method for detecting the presence of the 175 kDa form as a diagnostic marker of T cell activation. Quantitative determination of the presence of the 175 kDa form of DPPIV/CD26 can serve as
5 a precise measure of remaining disease activity following therapy to treat disorders of the immune system.

Therefore, the invention also features a method for determining the extent of T cell activation in a patient that includes detecting the presence of the 175 kDa form of DPPIV
10 in a biological sample. The method involves contacting the sample with an antibody capable of binding to the 175 kDa form of DPPIV, and not to the 105 kDa naturally occurring membrane form or the 105 kDa recombinant form of the protein, and detecting immune complex formation. Antibodies used in
15 the method of the invention, which can be intact antibodies or fragments thereof, can be polyclonal, or more preferably monoclonal, and can be labeled for ease of detection, i.e., directly labeled through coupling to a detectable substance (e.g., radioactive, enzymatic or fluorescent label) as well
20 as indirectly labeled through reaction with another reagent that is directly labeled. Examples of indirect labeling include, for example, detection of a primary antibody using a fluorescently labeled secondary antibody. In preferred
25 embodiments, the antibodies, fragments or derivatives are incorporated into immunoconjugates consisting of an antibody molecule or binding region thereof coupled (i.e., physically linked) to a detectable substance. Examples of detectable substances include various enzymes, prosthetic groups,

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fluorescent materials, luminescent materials, radioactive materials metal ions detectable by nuclear magnetic resonance, or other tracer molecule can be made by techniques known in the art. For instance, examples of suitable enzymes include horseradish peroxidase, alkaline phosphatase, B-galactosidase, or acetylcholinesterase; examples of suitable prosthetic group complexes include streptavidin/biotin and avidin/biotin; examples of suitable fluorescent materials include umbelliferone, fluorescein, fluorescein isothiocyanate; rhodamine, dichlorotriazinylamine fluorescein, dansyl chloride or phycoerythrin; an example of a luminescent material includes luminol; and examples of suitable radioactive materials include ^{125}I , ^{131}I , ^{35}S , or ^3H .

The term "biological sample" is intended to include tissue, cells and biological fluids isolated from a subject, as well as tissues, cells and fluids present *in vivo* within the subject. In preferred embodiments of the detection method, the biological sample is plasma, serum, urine, synovial joint fluid or cerebrospinal fluid, which following collection can be stored at temperatures below -20°C to prevent degradation until the detection method can be performed.

The detection methods of the invention can be used in immunohistochemical staining of tissue samples in order to evaluate the abundance of the 175 kDa form of CD26, or used diagnostically, e.g., in immunoassays, as part of a clinical testing procedure. Such immunoassays include, but are not limited to, competitive and non-competitive assay systems

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using techniques such as radioimmunoassays, ELISA, "sandwich" immunoassays, precipitin reactions, gel diffusion precipitin reactions, immunodiffusion assays, agglutination assays, complement fixation assays, immunoradiometric assays, fluorescent immunoassays, protein A immunoassays, and immunoelectrophoretic assays, to name but a few.

Such measurements can be useful in predictive evaluations of the onset or progression of immune disorders. Likewise, the ability to monitor the level of this protein in an individual can allow determination of the efficacy of a given treatment regimen for an individual afflicted with such a disorder.

The invention also encompasses kits for detecting the presence of the 175 kDa form of CD26 in a biological sample (e.g., plasma, serum or other biological sample as defined above). For example, the kit can comprise a labeled or labelable antibody, fragment or derivative which is capable of detecting the 175 kDa form of DPPIV in a biological sample; means for determining the amount of the protein in the sample; and means for comparing the amount of the 175 kDa form of CD26 in the sample with a standard (e.g., purified protein). The kit can be packaged in a suitable container, which can also include instructions for using the kit to detect the 175 kDa form of CD26.

Other features and advantages of the invention will be apparent from the following detailed description, and from the claims.

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BRIEF DESCRIPTION OF THE DRAWINGS

Fig. 1 shows the results of comparative electrophoretic analyses of rsCD26 and serum DPPIV/CD26 under various sets of conditions. Panel A: Analysis by SDS-PAGE (6%) under non-reducing conditions of rsCD26 (lane 1), and soluble serum DPPIV (lane 2). Panel B: Analysis by SDS-PAGE (10%) under reducing conditions of rsCD26 (lane 1), and soluble serum DPPIV (lane 2). Panel C: Analysis by Tris-Glycine Native - PAGE (7.5%) of rsCD26 (lane 1), and soluble serum DPPIV (lane 2);

Fig. 2 shows the results of analysis of glycosylation of rsCD26 (Lanes 1 - 4) and soluble serum DPPIV (Lanes 5 - 8) by sequential enzymatic deglycosylation and separated on a 10% SDS-PAGE gel under reducing conditions followed by silver staining. Lanes 1 and 5: untreated sample. Lanes 2 and 6: removal of terminal NeuAc with NANase II. Lanes 3 and 7: NANase II and removal of O-linked sugars by O-Glycosidase. Lanes 4 and 8: NANase II, O-Glycosidase and removal of N-linked sugars by PNGase F. Lanes 9 and 10: Periodic acid-Schiff staining for glycoprotein of the same rsCD26 samples as in Lane 1 and lane 4, and lanes 11 and 12: glycoprotein staining of the same serum DPPIV samples as in lanes 5 and 8;

Fig. 3 shows the results of analysis of epitope expression by rsCD26 (light stipple) and serum DPPIV (heavy stipple). Units of enzyme activity represent the DPPIV activity of the CD26/DPPIV bound to CD26-specific antibodies

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immobilized on anti-mouse Ig-Sepharose beads. UCHL1 (anti-CD45RO) and W6/32 (anti-HLA Class I) are control antibodies;

Fig. 4 shows ^3H -diisopropylfluorophosphate (^3H -DFP) binding to rsCD26 (■) and serum DPPIV (□). Each lane was cut into 2 mm slices, each of which was dissolved in scintillation fluid and counted. The abscissa represents distance (cms) traveled in the gel and is correlated with the indicated molecular weight markers which were run on the same gel. The ordinate (dpm) represents the amount of ^3H -DFP-labeled protein;

Figs. 5A-5B show the results of analysis of adenosine deaminase binding of rsCD26 and serum DPPIV/CD26. Panel A: Analysis of ADA-binding of rsCD26 and serum DPPIV detected by Surface Plasmon Resonance. ADA (10 $\mu\text{g}/\text{ml}$ in 10mM HEPES, 100 mM NaCl, 0.05% Tween 20) was passed over a Control carboxymethyl dextran (CM) surface (lowest trace), over serum DPPIV (middle trace), and over rsCD26 (upper trace), both immobilised on a CM surface. One thousand Resonance Units (ordinate) represents approximately 1 ng/mm^2 of binding protein. Panel B: Analysis of ADA-binding of rsCD26 and serum DPPIV by immobilization with anti-ADA. rsCD26 and serum DPPIV, incubated with or without ADA, were allowed to interact with rabbit anti-ADA or Normal Rabbit Serum (Control), followed by removal of immune complexes with Protein A-Sepharose beads. DPPIV/CD26 complexed to the beads was determined by DPPIV enzyme activity. The light stippled bars represent untreated, and the heavy stippled bars represent deglycosylated, rsCD26 and serum DPPIV;

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Fig. 6 shows T cell costimulatory activity developed by soluble DPPIV/CD26. rsCD26 (\square) and serum DPPIV (Δ) were incubated with PBL and tetanus toxoid in medium supplemented with soluble CD26-depleted autologous plasma. Proliferation was assayed by measurement of cell-incorporated ^3H -thymidine at 7 days. Vertical bars represent s.e.m. In the absence of tetanus toxoid, neither form of CD26/DPPIV had any effect upon the base response of 2351 ± 108 c.p.m. (data not shown);

Fig. 7 is a graph showing the DPPIV activity of material released from activated T cells compared to resting T cells. Purified T cells were incubated for the indicated time with or without PHA, and the DPPIV activity released into serum-free medium was determined. All DPPIV activity is associated with a 175 kDa molecule;

Fig. 8 shows the comparative ability of activated T cells, monocytes, macrophages and B cells to release DPPIV/CD26. T cells purified by sheep red blood cell rosetting technique (E-pos) were mixed with or without monocytes and B cells (E-neg) with or without PHA for the indicated periods in serum-free medium and the released DPPIV activity was determined; and

Fig. 9 shows the comparative ability of rsCD26, serum CD26/DPPIV and purified T cell-secreted DPPIV/CD26 to co-stimulate the response of normal T cells to recall antigen using similar conditions to those described in the legend to Figure 6.

DETAILED DESCRIPTION OF THE INVENTION

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We identify and describe here the properties and uses of, and useful poly and monoclonal antibodies specifically recognizing, a novel form of DPPIV/CD26 which is soluble in serum and distinct from the previously recognized membrane form. We show that this novel form of DPPIV exists naturally as a trimer with a monomeric molecular weight of 175 kDa, in contrast to rsCD26, which has a monomeric molecular weight of 105 - 110 kDa. Our novel form of DPPIV is probably a serine protease. Deglycosylation removed N-linked sugar from both our novel serum DPPIV and rsCD26 and no O-linked glycosylation was observed, revealing a protein core of 130 kDa for this 175 kDa serum form. The large 175 kDa form expresses functional DPPIV activity with similar substrate and inhibitor specificities and pH activity profile as rsCD26. Epitope analysis showed that monoclonal antibodies against five epitopes expressed by rsCD26 also bound, but more weakly, with 175 kDa DPPIV. Analysis of peptides after limited proteolysis and N-terminal sequencing revealed no homology with rsCD26 but some identity with other peptidases. Unlike rsCD26, the 175 kDa form does not bind ADA-1 and has no ADA-1 already associated with it. Similarly to rsCD26, the 175kDa form of DPPIV is a potent T cell costimulator. We conclude that the form of DPPIV we have found in human serum and plasma is unique and is not a breakdown product of membrane CD26. The conservation of DPPIV activity and five epitopes specific to rsCD26 suggest, however, a significant structural similarity.

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Purification of serum soluble DPPIV/CD26

The presence of soluble CD26 has been described previously in detergent-free cell extracts (23), and its presence has been inferred in serum from the identification of significant circulating DPPIV activity or by reactivity with defined anti-CD26 antibodies (15, 24 - 26). We have previously shown that serum soluble CD26/DPPIV may play an important role in regulating the immune response to recall antigens in vivo (15) and wished to confirm the identity of the serum form with the membrane form. Nevertheless, to clear serum of DPPIV activity using the high affinity anti-CD26 antibody 1F7 required large amounts of antibody, suggesting that there were structural differences between the membrane and soluble forms.

We thus set about purifying the DPPIV activity from human serum. Using both commercially available frozen serum as well as freshly isolated plasma, we developed a non-denaturing procedure for purifying the soluble DPPIV activity using techniques which were designed to maintain functional integrity. Although we had a large panel of antibodies available, we did not use these for affinity purification since we found that in every case the harsh elution conditions required (glycine-HCl, pH1 - pH2, or 3M KSCN) resulted in a reduction in the DPPIV specific activity of at least one order of magnitude. In every purification, the DPPIV activity and anti-CD26 reactivity was associated with a homogenous band of 175 kDa obtained by preparative native gel electrophoresis. In two preparations (out of 7 total),

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we were also able to copurify some lower molecular weight DPPIV activity corresponding to the 105 kDa form of CD26, but quantitation by laser densitometry confirmed that this was always less than 6% of the total preparation, and separated
5 away at the preparative native electrophoresis stage.

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Serum soluble DPPIV/CD26 is distinct from a shed form of 105kDa membrane CD26

Soluble DPPIV has been identified in pathological and normal human urine with a molecular weight of around 280 to 400 kDa determined by gel filtration (27, 28), although it is unlikely that the 400 kDa form represents a dimer of the serum form, since its size would not normally transfer by glomerular filtration. Kidney and epithelial-associated DPPIV/CD26 is a membrane dimer in the range 230 - 270 kDa (29) with reduced subunits of CD26 having a molecular weight of 105 kDa to 115 kDa, from which it is difficult to deduce any relationship with the serum form described here. One previous study purified an ADA-1 Complexing Protein (ADA-CP) of 105 kDa from human plasma (30), and their yield coincided with the small amounts of 105 kDa soluble CD26 we could isolate from some plasma samples (5 - 10 μ g/100 ml serum). Nevertheless, their procedure utilized a polyclonal antibody developed against ADA-CP, and purification was monitored by ADA activity which would miss the larger non-ADA-binding form of DPPIV.

This raises the issue of the origin of the large 175 kDa form of DPPIV we have found in serum. We have shown that the material in its reduced and deglycosylated form is larger than the membrane form. Therefore, it cannot result from shedding or proteolytic digestion of membrane CD26/DPPIV. Several mRNA species have been reported for human CD26 (11) although the evidence to date suggests that human DPPIV/CD26 is produced from a single location on chromosome 2 (31),

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perhaps allowing alternative splicing as a possible origin of serum CD26. One further possibility is that initiation occurs at two distinct promoter sites, as has already been shown for another membrane ectoenzyme, rat γ -glutamyl transpeptidase (32). There is also support for a distinct separate gene since DPP-X has been observed which shows great homology to DPPIV, but does not exhibit any dipeptidylpeptidase IV activity itself (33). Further evidence that there may be disparate genes coding for CD26-like molecules is provided by the recent report that Fibroblast Activation Protein- α (FAP- α) is a Type II membrane glycoprotein with remarkably similar sequence and organizational structure to membrane CD26 and, in fact, can form a heterodimer with membrane CD26 (34).

We may safely exclude the possibility that the DPPIV activity is the result of contaminating 105 kDa soluble CD26 or other aminopeptidases which may release the substrate chromophore by sequential degradation. The levels of aminopeptidase P in serum are essentially zero (25), while aminopeptidase M (CD13) activity could not be detected (by hydrolysis of Leu-pNA). Furthermore, the substrate specificities, inhibitor kinetics, and pH optimum of serum DPPIV were very similar to those of rsCD26.

Antigenic similarity between serum and membrane forms of DPPIV/CD26

Epitope analysis revealed that monoclonal antibodies against five epitopes expressed by rsCD26 also bound serum DPPIV. However, one of the differences between the serum and

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membrane forms was in the relative expression of epitopes detected by these monoclonal anti-CD26 antibodies. Although these epitopes were expressed by the large 175 kDa DPPIV, the antibodies bound more strongly to rsCD26. This made itself apparent particularly in a previous report by our group where very high concentrations of 1F7 antibody (40 μ g/ml) were required to clear DPPIV activity and CD26 antigenic activity from the serum (15). Nevertheless, the eventual complete clearance of DPPIV activity by this antibody, albeit at high concentrations, also implies that all the DPPIV activity in serum could be attributed to 1F7-reactive antigen and implies structural similarities between all functional DPPIV moieties in serum. In support, we have now produced a polyclonal rabbit antibody against purified serum DPPIV which binds strongly to Jurkat cells (CD26-negative) transfected with cDNA encoding the 105 kDa CD26/DPPIV (unpublished data). The N-terminal sequences of peptides released by protease treatment, however, suggest little sequence similarity between rsCD26 and serum DPPIV. Some caution must be exercised here since we may be comparing only those regions sensitive to proteolytic digestion; we have preliminary data that both proteins appear to have large protease-resistant regions. One recent report favors the notion that the DPPIV-related enzymes have arisen by divergent evolution from an α/β -hydrolase ancestor, where distinct secondary and tertiary structures may be retained despite complete differences in sequence (35). In contrast, the disparity of sequence and conservation of structure between the eukaryotic serine

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proteases, cysteine proteases, subtilisins and the α/β hydrolase fold enzymes provides a strong argument for convergent evolution (36).

Lack of affinity of serum soluble DPPIV/CD26 for ADA-1

5 A further difference between serum and recombinant CD26 concerns the inability of serum DPPIV to bind ADA-1. Human ADA exists as two forms, a low molecular weight (40 kDa) Type 1 form, and a high molecular weight (100 kDa) Type 2 form (37). ADA-1 accounts for almost all intracellular and
10 membrane-associated ADA while ADA-2 is predominantly found circulating in the serum. Recent reports show that ADA-1 interacts strongly with the membrane form of CD26 (13, 22), and that CD26 is equivalent to what was previously reported as ADA-CP. ADA-2 is not well characterized, but its levels
15 vary significantly with immune activity, particularly during opportunistic or systemic bacterial infections (38 - 40). There exists the possibility that serum DPPIV interacts with serum ADA-2 rather than with ADA-1, but this remains to be tested. Nevertheless, ADA-1 does not appear to play a role
20 in the T cell costimulatory activity mediated by serum DPPIV since the non-ADA-1-binding serum DPPIV is a potent costimulator of recall-antigen driven T lymphocyte responses in vitro.

Cellular origin of serum soluble DPPIV/CD26

25 The intriguing similarities and differences between the serum form and the membrane form of DPPIV/CD26 suggest a

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common origin of these glycoproteins. The clear ability of both DPPIV/CD26 forms to enhance immune function implies that both the 175 kDa and the 105 kDa forms of DPPIV/CD26 are important regulators of the in vivo immune response. We next investigated whether both forms are present in the T lymphocyte and determined that PHA-activated T cells release a soluble protein showing DPPIV activity, whereas such a protein is not released from resting T cells (Fig. 7). The released material has a molecular weight of 175 kDa and appears to be identical in other respects with serum soluble DPPIV/CD26. Only activated T cells release a protein showing DPPIV activity. Referring to Fig. 8, we have shown that a soluble DPPIV/CD26 is not released by E-negative cells, specifically monocytes, macrophages and B cells, and that the presence of such non-T immune cells does not enhance the release of 175 kDa DPPIV by T cells.

Kinetics of expression of 175 kDa DPPIV on the surface of activated T cells

We then used a polyclonal rabbit antibody against 175 kDa DPPIV (isolated as will be described below) to look for cells specifically expressing the large form, with the aim of identifying the site of origin serum DPPIV. By screening T cells at various stages of activation, other freshly isolated immune cells, T and B cell lines, and a large panel of carcinoma cell lines representing various epithelial and endothelial tissues, we were able to show that an antigen identical to serum DPPIV was coexpressed with 105 kDa CD26 on T cells and renal and pancreatic carcinoma cells. The

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kinetics of expression on activated T cells differed, however, since 105 kDa CD26 increases gradually during activation, concomitant with ADA-binding capacity, while 175 kDa DPPIV increased rapidly and peaked between days 2 and 3, after which levels fell. This led us to define 175 kDa DPPIV on the T cell membrane as a novel antigen, DPPT-L. We have subsequently shown that coincident with the fall in membrane expression, there was appearance of the 175 kDa form of DPPIV, DPPT-L, in the culture medium. Separation of T cells into subsets revealed that both CD4+ and CD8+ cells released this material and that within both these populations, the CD45RO+ subset was the predominant source.

We then compared the activation potential of the various forms of DPPIV/CD26 we have prepared and determined that rsCD26, purified serum/plasma 175 kDa DPPIV/CD26 and purified T cell-secreted soluble 175 kDa DPPIV/CD26 all can costimulate the response of normal T cells to the recall antigen, tetanus toxoid (Fig. 9). Our results show that the serum/plasma form and the T cell-secreted form of DPPIV/CD26 are significantly more effective than the recombinant soluble form at enhancing the immune response in vitro, but nevertheless showed the identical kinetics of costimulation to those displayed by recombinant soluble CD26.

Isolation of antibodies capable of distinguishing the 175 kDa form of DPPIV/CD26 from the 105 kDa form

In order to distinguish the 175 kDa form of DPPIV/CD26 from the 105 kDa form, we first isolated a polyclonal

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antibody that can bind specifically to 175 kDa DPPIV/CD26. Following multiple immunizations of rabbits with the 175 kDa form of DPPIV/CD26, the rabbit serum was passed onto a Protein A-Sepharose column to bind the immunoglobulin fraction, which was then eluted. The purified immunoglobulins contained antibody which could bind to cells expressing only the 105 kDa DPPIV/CD26 as well as to activated T cells expressing both the small and large forms of DPPIV/CD26. The purified immunoglobulin preparation was then passed over a Sepharose column to which had been immobilized recombinant soluble CD26. This procedure removes all the antibody directed against the 105 kDa DPPIV/CD26. The material passing through the column without binding was then tested again against a cell line expressing only the 105 kDa form, and no binding was observed. Nevertheless, the antibody could still bind to activated T cells expressing the large 175 kDa form, and this was confirmed by the ability of the antibody to immunoprecipitate the 175 kDa molecule with minimal immunoprecipitation of the soluble 105 kDa form. These results show that while the 175 kDa DPPIV/CD26 and 105 kDa DPPIV/CD26 share many common antigenic epitopes, the 175 kDa DPPIV/CD26 also includes some unique epitopes not expressed on the 105 kDa DPPIV/CD26.

In addition, as will be described in more detail below, we have also prepared a panel of monoclonal antibodies specifically directed against the 175 kDa form of DPPIV. These antibodies were selected for their ability to react with 175 kDa DPPIV and failure to react with 105 kDa

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CD26/DPPIV utilizing a screening procedure that distinguished between binding to purified serum 175 kDa DPPIV and binding to rsCD26. The resultant mAb (4B3, 1D1, 1D2, 1D3, 1B6, 1A6 and 5D2), like the rabbit anti-175 kDa DPPIV, are minimally reactive with freshly isolated unactivated T cells, unreactive with Jurkat transfectants expressing the 105 kDa CD26 and are strongly reactive with 175 kDa DPPIV on activated T cells.

Isolation of nucleic acid encoding the 175 kDa form of DPPIV/CD26

We will isolate a cDNA clone encoding 175 kDa DPPIV/CD26 by a simple and highly efficient cloning strategy designed for human lymphocyte glycoproteins, which is based on transient expression in COS cells, a technique initially devised by Aruffo and Seed (Proc. Nat. Acad. Sci. USA 84:8573-7, 1987). Central to this strategy is the use of an efficient method to prepare large plasmid cDNA libraries. The libraries are introduced into COS cells, which transiently express the transfected cDNA and can bind to solid phase immobilized anti-175 kDa DPPIV antibody. The non-binding cells (not expressing 175 kDa DPPIV) are washed away, and plasmid-cDNA is isolated from the cytoplasmic extracts of the antibody-immobilized cells. This plasmid cDNA is then used to transfect a further population of COS cells, and isolation of 175 kDa DPPIV expressing cells followed by extraction of plasmid cDNA is performed repeatedly (usually 3-5 rounds) until it is apparent that the

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cdNA of interest has been enriched sufficiently to allow preparation of individual transformed bacterial colonies for amplification of the cdNA and subsequent sequencing. Other cloning strategies which are designed to detect glycoproteins, especially panning selection procedures as described above, could also be used to isolate a cdNA clone encoding the novel glycoprotein of the invention (e.g., see Frohman et al., "Rapid amplification of complementary DNA ends for generation of full-length complementary cDNAs: Thermal RACE," Methods. Enzymol. 218:340-358, 1993).

The following examples are presented to illustrate the advantages of the present invention and to assist one of ordinary skill in making and using the same. These examples are not intended in any way otherwise to limit the scope of the disclosure.

EXAMPLE I

Purification of serum DPPIV

DPPIV from serum was isolated by sequential purification using ion exchange chromatography (s-Sepharose), ConA affinity chromatography, and a second separation on s-Sepharose. Approximate levels of purification and yields are presented in Table 1. The chromatographic procedures yielded a preparation which displayed three protein bands by SDS/PAGE under reducing conditions. None of the bands co-migrated with the 105 kDa rsCD26. The preparation was further purified by eluting the three bands from a non-denaturing native preparative Tris-Glycine gel. All DPPIV enzyme activity was

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associated with a single high molecular weight band on the native gel which consisted predominantly of a 175-180 kDa moiety by non-reducing and reducing SDS-PAGE (Figure 1A, B). Under native conditions, the serum DPPIV activity migrated differently from rsCD26 (Figure 1C). This preparation was further analyzed by matrix-assisted laser desorption mass spectrometry (21) which revealed a strong peak at approximately 175 kDa while rsCD26 produced a strong signal at 101 - 105 kDa. In addition, gel filtration chromatography on Superdex 200 revealed that in its native state, the serum DPPIV migrates between thyroglobulin (669 kDa) and apoferritin (443 kDa) as a large complex of 570 kDa. Since the material applied to the gel filtration column was not appreciably contaminated by any other protein, the serum DPPIV would appear to exist as a trimer. Due to availability, the DPPIV was purified from human serum rather than plasma. Nevertheless, purification of DPPIV from 50 ml plasma of 7 normal donors revealed an identical 175 - 180 kDa band. In two instances, the 105 kDa form copurified with the 175 kDa form, but was less than 6% of the total protein preparation by laser densitometry.

EXAMPLE II

Biochemical characterization of serum CD26/DPPIV

The membrane form of CD26 is heavily glycosylated (approximately 16% of the molecular weight (18) so we examined the pattern of glycosylation of the serum form to determine whether it resulted from hyper-glycosylation of the identical peptide backbone found in the membrane form.

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Accordingly, samples of both rsCD26 and serum DPPIV were sequentially digested by neuraminidase (NANase II), by O-glycosidase, and then by PNGase F (removing NeuAc, O-linked and all N-linked oligosaccharides and poly-sialic acids). As shown in Figure 2, the pattern of digestion is similar for rsCD26 and serum DPPIV, demonstrating that both have significant N-linked glycosylation but little if any O-glycosylation. Nevertheless, the deglycosylated backbone of serum DPPIV remains larger than that of rsCD26 (approximately 130 kDa in comparison with 90 kDa, the latter figure agreeing well with the 89 kDa predicted from the amino acid sequence), demonstrating that the higher molecular weight of the serum form is not due to hyperglycosylation. Staining for glycoprotein revealed that enzymatic deglycosylation resulted in removal of all carbohydrate residues detectable by periodic acid-Schiff's reagent (Figure 2, lanes 9 - 12).

EXAMPLE III

Enzymatic properties of serum CD26

The DPPIV enzyme activities of the rsCD26 and serum DPPIV were analyzed by Michaelis-Menten kinetics by assessing kinetics of hydrolysis of substrate at different concentrations. Analysis using the Lineweaver-Burke transformation yielded an identical K_m of 0.28 mM for Gly-Pro-pNA for both recombinant and serum DPPIV and the values for V_{max} of 1.52 U/nmol rsCD26 and 0.65 U/nmol serum DPPIV were in the same range, using Gly-Pro-pNA as substrate. Inhibition by the specific DPPIV inhibitor Diprotin A was similar for both serum DPPIV and rsCD26, with an IC_{50} of 0.125

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mM for rsCD26, and 0.25mM for serum DPPIV. To further exclude the possibility that we had purified irrelevant aminopeptidases which, working in concert or by sequential hydrolysis, could release the pNA chromophore from substrate, we tested a number of substrates which could be hydrolyzed by non-DPPIV aminopeptidases. One candidate for sequential hydrolysis is aminopeptidase M (3.4.11.2), but no hydrolysis of Ala-pNA, Arg-pNA or Leu-pNA was observed and activity by aminopolypeptidase (3.4.11.14) may be similarly excluded. The substrate succinyl-Gly-Pro-pNA was also not hydrolyzed. The only substrate apart from the dipeptide chromatogen Gly-Pro-pNA which could be hydrolyzed was Val-Ala-pNA, which was cleaved by both the serum DPPIV and rsCD26 (relative to rate of hydrolysis of Gly-Pro-pNA, hydrolysis of Val-Ala-pNA was 0.098 and 0.079, for rsCD26 and serum DPPIV, respectively). Although DPPIV primarily hydrolyses dipeptides with a proline in the penultimate position, it is well documented that alanine in the penultimate position may also render a dipeptide sequence susceptible to cleavage by DPPIV (8). The Gly-Pro-pNA substrate may also be a substrate for dipeptidylpeptidase II (DPPII), as may Val-Ala-pNA, but this enzyme has an acidic pH optimum (8). We determined the pH optima for both the rsCD26 and the serum DPPIV and showed them to be identical at pH 8.5, while there was almost a complete inhibition of activity at acidic pH 4.5-5, suggesting that we were not purifying a DPPII-like molecule.

EXAMPLE IV Epitope analysis

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In order to compare the antigenic sites of rsCD26 and serum DPPIV, we used a panel of monoclonal anti-CD26 antibodies developed in our laboratory which had been shown to detect 5 distinct antigenic epitopes of CD26 by cytofluorimetry and by cross-blocking using Surface Plasmon Resonance. Both serum DPPIV and rsCD26 were incubated with the murine monoclonal anti-CD26 antibodies (1F7, 5F8, 9C11, 2F9, and 4G8 which detect distinct epitopes, and 10F8A which detects an epitope shared by the 1F7 and 2F9 determinants) and with murine monoclonal control antibodies (W6/32 and UCHL1) after which the antibody-antigen complexes were removed from unbound material by binding to anti-mouse IgG Sepharose beads. Complexed CD26 was determined by DPPIV activity which was proportional to the amount of CD26/DPPIV immobilized (Figure 3). The serum DPPIV and the rsCD26 both bind 1F7, 10F8A, 5F8, 4G8 and 9C11. The complete panel of anti-CD26 antibodies can bind to both serum DPPIV and rsCD26, and despite the binding being greater to the recombinant form, the implication is that the two proteins share some structural motifs.

EXAMPLE V

N-terminal sequencing of tryptic peptides of serum DPPIV

N-terminal sequences of 10 peptides derived after tryptic digestion of reduced and alkylated serum DPPIV were determined. Of these, none revealed a complete identity with any sequence within available computerized databases. Nevertheless, 6 of the peptides revealed significant homology and similarity (using conservative substitutions) to a

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metalloendopeptidase (leishmanolysin), a serine endopeptidase (myeloblastin), a protein classified originally as a proteolytic plasminogen activator (neutral proteinase, of which the protease activity is now questionable), urokinase plasminogen activator, and chymotrypsin-like serine protease precursor.

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EXAMPLE VI
Serum DPPIV is a serine proteinase

To determine the functional classification of serum DPPIV and compare it with rsCD26 which is known to be a serine proteinase, we incubated the serum DPPIV with ^3H -DFP which binds to serines in the active site of serine proteinases. As shown in Figure 4, the serum DPPIV could be labeled with DFP and formed a sharp peak around 175-180 kDa, while the rsCD26 was more broadly spread over the region of 105 - 115 kDa, which might be attributed to heterogenous glycosylation (18).

EXAMPLE VII
Binding of ADA-1 to CD26

rsCD26 has been shown previously to be identical to the membrane protein which binds ADA-1 (13, 22). We used two techniques to determine whether serum DPPIV binds ADA-1. The first technique utilized Surface Plasmon Resonance to measure the interaction of ADA-1 with serum or recombinant CD26 immobilized on a carboxymethyl dextran surface. It is clear that the rsCD26 has a high affinity for ADA-1 but that this is not so for serum DPPIV (Figure 5A) which shows a binding profile similar to that of the Control. To confirm this, we utilized a technique whereby rabbit anti-ADA antibodies were incubated with rsCD26 or serum DPPIV, and the immune complexes formed were removed with Protein A Sepharose beads. Binding of ADA-1 to CD26 was then confirmed by detection of DPPIV activity associated with the extensively washed beads. In the absence of exogenously-added ADA-1 (Figure 5B, " -

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ADA"), neither the rsCD26 nor the serum CD26 had any associated ADA-1 prior to the experiment. Upon addition of exogenous ADA (Figure 5B, " + ADA"), only the rsCD26 bound to the ADA, and no evidence of ADA-binding to serum DPPIV was observed. Although the rsCD26 DPPIV activity developed within 10 minutes of adding substrate, the samples were left for 24 hr after which time the samples incubated with serum CD26 had still not developed a signal above background, showing that the lack of ADA-binding was absolute, and not a relative difference. It is also of interest to note that deglycosylated rsCD26 is still able to bind ADA-1 as well as the untreated form, suggesting that the ADA binding site on rsCD26 is not in the heavily glycosylated region proximal to the membrane.

EXAMPLE VIII

T cell costimulatory activity of CD26

We have previously shown that rsCD26 is able to enhance the responses of peripheral blood leukocytes (PBL) to the recall antigen, tetanus toxoid (15). Accordingly, we tested whether serum DPPIV could also enhance the responses of PBL to tetanus toxoid. As shown in Figure 6, the serum DPPIV was as capable as rsCD26 at costimulating the response to tetanus toxoid, with the peak response in the range 0.5 to 1 $\mu\text{g/ml}$. As we have routinely observed, concentrations of rsCD26 higher than 1 $\mu\text{g/ml}$ tend to be inhibitory, and a similar phenomenon was observed for serum DPPIV. It is important to note here that all autologous plasma samples used as culture supplements during the incubation with recall antigen were

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precleared of endogenous CD26/DPPIV activity by pretreatment of the plasma samples with anti-CD26 antibody and removal of immune complexes with anti-mouse-IgG magnetic beads.

EXAMPLE IX

5 Production of monoclonal antibodies against
 serum 175 kDa DPPIV (DPPT-L)

Balb/C mice were immunized intraperitoneally (i.p.) with 50 µg purified human serum DPPT-L in Complete Freund's Adjuvant, followed by biweekly booster injections i.p. of 50 µg purified human serum DPPT-L in Incomplete Freund's Adjuvant. An Enzyme-Linked Immunosorbent Assay (ELISA) was used to check that individual immunized mice were producing murine polyvalent antibody against human DPPT-L. Three days after the final boost, spleen cell suspensions were prepared and were fused with NS-0 murine myeloma cells using the polyethylene glycol fusion technique first described by Kohler and Milstein (European Journal of Immunology 6:511-519 (1976)). Fused cells were plated out in 96 well microtiter plates and viable hybrids were selected by their ability to grow in the presence of aminopterin. Supernatant from wells containing single actively-growing colonies was screened by ELISA for murine Ig production and, if positive, were then screened for specificity of binding using an ELISA detecting binding to recombinant CD26 and purified DPPT-L. Clones secreting antibody which preferentially bound to DPPT-L but did not detect epitopes shared by DPPT-L and CD26 were isolated, expanded and subcloned by limiting dilution to ensure clonality. Secreted antibody was purified from bulk

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tissue culture supernatant and from ascites grown in pristane-primed mice. Of 24 antibodies characterized, all were IgM. The antibodies were checked for ability to bind to 48 hr-activated T cells but not resting T cells and seven (4B3, 1D2, 1D1, 1B6, 1A6, 1D3 and 5D2) were chosen on the basis of strongest antigen-binding capacity for use in further structural and functional characterization of DPPT-L.

Materials and Methods

Recombinant soluble CD26: The full extracellular domain of T cell membrane-expressed CD26 (rsCD26) was obtained as a soluble secreted product from transfected CHO cells as described previously (15). The transfected CHO cells were grown in serum-free CHO-SFM medium to confluence (Life Technologies Inc., Gaithersburg, MD). Sodium azide (0.1% w/v final) was added to the rsCD26-containing conditioned medium which was passed at 1 ml/min over a 38 x 1.5 cm concanavalin A (ConA) - agarose column (Sigma, St Louis, MO) equilibrated in 2x phosphate-buffered saline (2x PBS; 30.8 mM NaCl, 0.54 mM $\text{Na}_2\text{HPO}_4 \cdot 7\text{H}_2\text{O}$, 0.31 mM KH_2PO_4 ; pH6.8) at room temperature (as were all subsequent column procedures). After washing with 5 column volumes of 2x PBS, bound glycoprotein was eluted with 2x PBS containing 200 mM α -methylmannoside (Sigma) and dialyzed extensively against 50 mM sodium acetate buffer, pH4.65. After equilibrating a 8.5 x 2.5 cm s-Sepharose (Pharmacia, Piscataway, NJ) ion exchange column in 50 mM sodium acetate, pH4.65 (equilibration buffer), the DPPIV-active ConA eluate was then loaded at 1 ml/min. The column

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was then washed with 10 column volumes of equilibration buffer and eluted with a 0 - 1M NaCl gradient in equilibration buffer. The rsCD26 elutes as a clean peak at 50 mM NaCl and all eluted DPPIV activity was associated with this peak.

DPPIV activity: The primary substrate used for determining DPPIV activity in all assays was Gly-Pro-p-nitroanilide (Gly-Pro-pNA; Sigma), which is hydrolyzed by DPPIV to release pNA absorbing strongly at 405 nm. Kinetic assays were performed using varying amounts of substrate in order to obtain estimates of the K_m and V_{max} using standard Michaelis-Menten kinetics. For screening assays of DPPIV activity, 150 ml of substrate was used at 1 mg/ml (2mM final concentration) in phosphate buffer (pH7.6, 100 mM) in 96 well flat-bottomed microtiter plates together with 10 ml of appropriately diluted sample. For samples obtained after elution from the s-Sepharose column, the pH was raised to pH7.6 by addition of 25 μ l of 1M Tris-HCl, pH7.6 to the reaction mixture. One unit (U) of enzyme activity was defined as the amount of enzyme that cleaved 1 mmol of Gly-Pro-pNA/min at pH7.6 and 20°C. Inhibition of the hydrolysis of Gly-Pro-pNA was determined in the presence of the specific DPPIV inhibitor Diprotin A (Bachem, King of Prussia, PA). To test for the presence of non-DPPIV peptide hydrolase activity, the following substrates were used (all obtained from Bachem): Leu-pNA, Ala-pNA, Arg-pNA, succinyl-Gly-Pro-pNA, Gly-Arg-pNA, and Val-Ala-pNA (a weak DPPIV substrate). The pH optimum was

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determined substituting phosphate buffer (100 mM, pH4.5 - pH9) in place of standard incubation buffer.

Serum DPPIV: Serum DPPIV was prepared by isolating from 100 ml of pooled normal human male AB serum the fraction of protein precipitated by 50 - 70% saturated ammonium sulfate. After extensive dialysis against 50 mM acetate buffer, pH4.65, the material was passed onto, and eluted from, a s-Sepharose column in an identical manner to that described above for rsCD26. The DPPIV-positive fractions were pooled and dialyzed against 2x PBS prior to binding and elution from a ConA-agarose column as described above for rsCD26. The ConA eluate was dialyzed against acetate equilibration buffer and re-applied to the s-Sepharose column which was then washed with 10 volumes of equilibration buffer. The DPPIV activity was eluted by a 0 - 0.5M NaCl gradient in equilibration buffer and all DPPIV activity was found in the 10 mM - 50 mM NaCl eluate (determined by conductance). The eluted material was then concentrated to 1 - 2 ml using 10 kDa cut-off centrifugal concentrators (Centriprep 10; Amicon, Beverley, MA) and was then applied to a preparative native 7.5% acrylamide gel buffered with Tris-Glycine. After electrophoresis, the gel was cut horizontally into 1 mm strips and the >30 kDa material was extracted from the gel slices by electroelution (Model 422; BioRad, Melville, NY). The eluted material expressing DPPIV activity was characterized by running on a 7.5% SDS-PAGE gel under reducing conditions followed by silver staining (Amersham,

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Arlington Heights, IL). The molecular weight of serum DPPIV was also determined by its application (50 μ g in 100 ml PBS) on to a 100 x 1.5 cm Superdex 200 column (Pharmacia) equilibrated in PBS after calibration using Blue Dextran (for void volume), thyroglobulin (669 kDa), apoferritin (443 kDa), alcohol dehydrogenase (150 kDa) and bovine serum albumin (66 kDa), all obtained from Sigma. Flow rate was 0.4 ml/min, protein elution was monitored by optical density at 280 nm, and the DPPIV-containing fractions were localized by ability to hydrolyze Gly-Pro-pNA using the standard conditions described above.

Enzymatic deglycosylation: Sequential deglycosylations were performed using recombinant N-acetylneuraminidase II (NANase II; 10 mU/10 μ g substrate protein), O-glycosidase (2 mU/10 μ g substrate protein), and Peptide:N-glycosidase F (PNGase F; 5 mU/10 μ g substrate protein) using buffers and reagents supplied (Glyko, Novato, CA). Briefly, the NANase II (removing a[2-3]-, a[2-6]-, and a[2-8]-linked NeuAc) and O-glycosidase digestions were performed for 1 hr at 37°C in 50 mM sodium phosphate, pH6.0. The pH of the samples was then raised by addition of Na₂HPO₄ to 0.125M final, pH8.0, followed by boiling in the presence of SDS and β -mercaptoethanol for 5 min to denature the protein. Nonidet P-40 (NP-40, 2.5 μ l/40 μ l sample) was added prior to incubation with PNGase F for 3 hr at 37°C which removed N-linked tri- and tetra-antennary complex-type oligosaccharide chains, poly-sialic acids, and high-mannose and hybrid-oligosaccharide chains. The samples

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were again incubated with SDS-PAGE reducing sample buffer at 100°C prior to electrophoresis on 10% acrylamide gels. To check for complete deglycosylation, the enzyme-treated proteins were stained for carbohydrate moieties in 10% SDS-PAGE gels by oxidation with periodic acid followed by staining with Schiff's reagent (Sigma).

Epitope analysis: 1 µg of serum DPPIV or rsCD26 was incubated in 100 µl of PBS containing 0.05% Tween 20 (Sigma; PBS-Tween) together with 5 µg purified Control or anti-CD26 antibodies for 1 hour at 4°C with rotation. Control murine monoclonal antibodies used were W6/32 (anti-β2-microglobulin/HLA Class I, IgG₂, ref. 16) and UCHL1 (anti-CD45RO, IgG_{2a}, ref. 17). The anti-CD26 murine monoclonal IgG antibodies used were 1F7 (IgG₁) and 5F8 (IgG₁) described previously (2, 18) and 10F8A, 9C11, 4G8, and 2F9 (all IgG₁) which were produced in our laboratory after immunization of mice with CD26-transfected 300.19 cells, a murine pre-B cell lymphoma line. All antibodies were purified by binding to and elution from Protein A-Sepharose (Pharmacia). By epitope analysis using Surface Plasmon Resonance (BIAcore, Pharmacia Biosensor AB), 5F8, 2F9, 4G8, 9C11 and 1F7 detected 5 unique epitopes while 10F8A was blocked by 1F7 and 2F9. Anti-mouse IgG-agarose beads (Sigma; 50 µl) were added to each sample and incubated for a further 16 hr at 4°C with rotation followed by 5 washes of the beads with PBS-Tween to remove unbound CD26 and antibody. Twenty µl of the washed beads were then incubated in duplicate together with Gly-Pro-pNA (2 mM final

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concentration). The enzyme activity, determined as hydrolysis and release of pNA measured at 405 nm, of the antibody-immobilized CD26/DPPIV provided a relative measure of the affinity of the particular antibody for CD26.

5 Peptide and Sequence Analysis: For N-terminal sequencing, 50 µg (~280 pmol) of serum DPPIV was electrophoresed on a 7.5% SDS-PAGE gel, electrotransferred to a PVDF membrane (0.45 mm; BioRad), and stained with 0.1% Ponceau S in 5% acetic acid (Sigma). After cutting out the stained band at 175 - 180
10 kDa, protein was eluted from a small piece of membrane for amino acid analysis to estimate the amount of transferred protein. The protein was then subjected to tryptic digestion on the PVDF membrane and the products were resolved by narrow-bore reverse phase HPLC as described (19). Peaks
15 suitable for N-terminal sequencing were selected, checked for molecular weight by matrix-assisted laser desorption mass spectroscopy and sequence determined using a Model 477A Protein Sequencer linked to a Model 120A PTH-amino acid analyzer (Applied Biosystems, Foster City, CA). All sequence
20 analysis was performed using the GCG Unix package (Genetics Computer Group, Madison, WI).

Affinity-labeling of enzyme active site: To determine whether serum DPPIV is a serine protease similar to rsCD26, the active sites of both proteins (5 µg in 20ml 100 mM Tris-HCl, pH8.0) were labeled with 25 µCi ³H-di-isofluorophosphate
25 (DFP, 1 mCi/ml; New England Nuclear, Boston, MA) for 24 hr

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at 37°C after which the proteins were separated by SDS-PAGE (7.5%). The gel was fixed in 25% isopropanol/10% acetic acid and 2 x 10 mm slices of the lanes containing radiolabeled sample were cut out, homogenized in 1.5 ml polypropylene tubes and the slurries were incubated with 1 ml of scintillation cocktail (Atomlight; New England Nuclear) for 5 days with occasional shaking until all material had entered into solution. Further scintillation fluid (2 ml) was added and the samples were counted in a b-scintillation counter.

CD26 Binding to Adenosine Deaminase (ADA-1): Purified serum DPPIV or rsCD26 (50 µg/ml in 20 mM acetate buffer, pH 4.5) was immobilized on the carboxyl-methyl dextran surface of a CM5 Sensor chip by N-hydroxysuccinimide/carbodiimide-mediated amine coupling (BIAcore, Pharmacia Biosensor AB). The ability of bovine adenosine deaminase (ADA-1, 10 µg/ml in 10 mM HEPES pH7.4, 100 mM NaCl, 0.05% Tween 20) to bind to the CD26-coated surface was determined by measuring Surface Plasmon Resonance (20). In a separate technique, and to simultaneously determine whether adenosine deaminase was already bound to serum DPPIV, samples of serum DPPIV and rsCD26 (1 µg/ml) were incubated for 30 min at 4°C in the presence or absence of ADA-1 (10 µg/ml in Tris Buffered Saline, pH7.4, containing 0.1% Tween; TTBS), followed by an incubation with a polyclonal rabbit anti-human ADA (Dr J. Kameoka, Dana Farber Cancer Institute; diluted 1:20 in TTBS) or normal rabbit serum control. Protein A-Sepharose (Sigma; 50 µl) was added and the samples incubated overnight while

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rotating at 4°C. After 4 washes in TTBS, the Protein A-Sepharose beads were tested for DPPIV activity by incubation with Gly-Pro-pNA (2 mM final concentration).

Proliferation Assays: Peripheral blood leukocytes were isolated from donors tested previously for positive proliferative responses to tetanus toxoid, where such responses could be further enhanced by addition of rsCD26. PBL were resuspended at 5×10^4 in 200 μ l RPMI 1640 containing 10 % autologous serum depleted of endogenous CD26 by batch incubation with anti-CD26 antibodies as described previously (15), with or without tetanus toxoid (Connaught Laboratories, Swiftwater, PA; 10 μ g/ml) and serum DPPIV or rsCD26 at the indicated concentrations and incubated in round-bottomed 96 well microtiter plates for 7 days at 37°C in a humidified 7.5% CO₂ atmosphere. Each well was pulsed with 1 μ Ci tritiated thymidine (New England Nuclear) and was incubated for a further 24 hr before harvesting and counting incorporated thymidine using a β -scintillation counter.

USE

Soluble serum DPPIV/CD26 and variants thereof are generally useful as immune response-stimulating therapeutics as described in U.S. Pat. Appl. Ser. No. 08/388,221, the whole of which is incorporated by reference herein. For example, the compounds of the invention can be used for treatment of disease conditions characterized by immunosuppression: e.g., AIDS or AIDS-related complex, other

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virally- or environmentally-induced conditions, and certain congenital immune deficiencies. The compounds may also be employed to increase immune function that has been impaired by the use of immunosuppressive drugs such as certain chemotherapeutic agents, and therefore are particularly useful when given in conjunction with such drugs. When given as an adjuvant in conjunction with a vaccine antigen, the compounds of the invention will boost the immune response triggered by the vaccine and thus increase the vaccine's protective potency. This would be particularly beneficial where the vaccinee is incapable of generating an optimal immune response without the use of such an adjuvant, as is the case for newborns or for persons undergoing renal dialysis or transplantation, or where the vaccine antigen is one which is poorly immunogenic.

Generally, the compounds of the invention will be suspended in a pharmaceutically-acceptable carrier (e.g., physiological saline) and administered orally or by intravenous infusion, or injected subcutaneously, intramuscularly, or intraperitoneally. Optimal formulation and dosage can be readily determined by one of ordinary skill in the art of pharmacology, taking into account such factors as the biological half-life of the compound and the degree of immunostimulation desired. It is expected that a typical dose for a severely immunocompromised patient will be approximately 0.01 to 100 $\mu\text{g/kg/day}$. When utilized as a vaccine adjuvant, a typical single dose of the compound of the invention would be 0.1 to 100 μg .

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Alternatively, peripheral blood lymphocytes can be withdrawn from the patient and treated with the serum DPPIV/CD26 compound of the invention (whether in soluble form or attached to a solid support by standard methodologies) ex vivo, prior to introducing the newly-stimulated lymphocytes into the same or a different patient.

Antibodies directed against the 175 kDa form of DPPIV, particularly monoclonal antibodies, are generally useful for differentiating between serum/plasma DPPIV activity attributable to enzyme released from activated T cells; and DPPIV activity released from damaged endothelium and epithelium. This is an important distinction since serum DPPIV has been shown to change dramatically in pathological processes such as systemic lupus erythematosus (SLE) and rheumatoid arthritis (RA) where both kidney epithelium damage and profound T cell activation coexist. The ability to monitor specifically one further measure of T cell activation, e.g., in plasma, serum, synovial fluid or cerebrospinal fluid, provides a handle for assessing immunomodulatory therapies administered for SLE, RA and other autoimmune diseases such as multiple sclerosis and could potentially be applied to other immunopathologies where alterations in T cell activity are observed. Specifically, plasma or serum DPPIV can be used, together with other tests for characterizing T cell subsets, to monitor the recovery of T cells after bone marrow transplantation procedures and simultaneously provide a measure of the efficacy of immunosuppressive protocols designed to prevent Graft-versus-

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Host Disease, characterized by activated T cells. In contrast, the measurement of T cell-released 175 kDa DPPIV can provide a sensitive marker of the stages of T cell activation and subsequent loss during the development of AIDS, and, further, be used to monitor potential therapies designed to maintain T lymphocyte levels in HIV seropositive individuals.

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Table 1: Purification of CD26/DPPIV from normal human serum¹

	Protein (mg)	Total Activity ² (units)	Specific Activity (units/mg)	Yield (%)	Fold Puri- fication
5	Original serum	8900	3.47 x 10 ⁻⁴	100	1
	50-70% Ammonium Sulfate Cut	2648	8.53 x 10 ⁻⁴	73.1	2.46
	s-Sepharose	303	5.76 x 10 ⁻³	56.5	16.58
	concanavalin A	12.49	0.115	46.4	330.6
10	s-S pharose	3.01	0.413	40.2	1188
	Preparative Native Electro- phoresis	0.175	1.78	10.1	5125

1 100 ml of normal human male AB serum

15 2 One unit is defined as the amount of enzyme cleaving 1 mmol of Gly-Pro-pNA/min under the assay conditions used.

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Table 2: N-terminal sequences determined for serum DPPIV after proteolytic digestion. Dashes (--) indicate N-terminal or C-terminal extensions, a single dash (-) indicates that a gap has been inserted for alignment. A 'x' indicates that an amino acid could not be reliably identified at that position. Similarity was determined by conservative replacement using the 'FASTA' program (Genetics Computer Group, Madison, WI).

			<u>% Identity/- Similarity</u>
10	1. serum DPPIV 3.4.24.36 ^a	LTGSSGFTD GPGNYK --VRGSSGYVAC TPGQRV--	44/75
	2. serum DPPIV 3.4.21.76 ^b	YDQDTQMxTI LK --YDAENKLNDI LL--	33/92
15	3. serum DPPIV Protease III ^c	GFNLIIT --GFNLITK--	71/71
	4. serum DPPIV 3.4.21.73 ^d	QNALLARLT MRALLARLL--	67/67
	5. serum DPPIV 3.4.21.73	GDQHTDCYSC TAN-TNDCHW C --PQNERQCYSC KGNSTHGCSS E--	30/57
20	6. serum DPPIV 3.4.21.- ^e	SGQISIFG --SGHICIFE--	63/68

^a EC 3.4.24.36: leishmanolysin precursor (cell surface protease)

^b EC 3.4.21.76: myeloblastin (proteinase 3 precursor, Wegener's autoantigen)

^c Protease III: originally classified as EC 3.4.24.4 (neutral proteinase).

^d EC 3.4.21.73: urokinase plasminogen activator

^e EC 3.4.21.- : chymotrypsin-like serine proteinase precursor

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Abbreviations:

	ADA-1	adenosine deaminase type I
	ADA-CP	adenosine deaminase complexing protein
	ConA	concanavalin A
5	DFP	di-isopropylfluorophosphate
	DPPII	dipeptidylpeptidase II
	DPPIV	dipeptidylpeptidase IV
	NANase II	recombinant neuraminidase II
	NP-40	Nonidet P-40
10	PBL	peripheral blood leukocytes
	PBS	phosphate-buffered saline
	pNA	p-nitroanilide
	PNGase F	recombinant Peptide:N-glycosidase F
	PVDF	polyvinylidene difluoride
15	rsCD26	recombinant soluble CD26
	SDS-PAGE	SDS polyacrylamide gel electrophoresis

References

1. Fox et al., J. Immunol. 133:1250-1256 (1984).
2. Morimoto et al., J. Immunol. 143:3430-3439 (1989).
3. Hafler et al., J. Immunol 137:414-418 (1986).
- 5 4. Stein et al., "Leukocyte Typing IV" (Knapp, W., Oxford University Press, Oxford, Great Britain (1989).
5. Hegen et al., J. Immunol 144:2908-2914 (1990).
6. Ulmer et al., Scand. J. Immunol. 31:429-435 (1990).
- 10 7. Tanaka et al., Proc. Natl. Acad. Sci. USA 90:4586-4590 (1993).
8. Yaron et al., Crit. Rev. Biochem. Mol. Biol. 28:31-81 (1993).
9. Heymann et al., FEBS Lett. 91:360-364 (1978).
10. Dang et al., J. Immunol. 144:4092-4100 (1990).
- 15 11. Tanaka et al., J. Immunol. 149:481-486 (1992).
12. Torimoto et al., J. Immunol. 147:2514-2517 (1991).
13. Kameoka et al., Science 261:466-469 (1993).
14. Buc et al., Eur. J. Immunol. 20:611-615 (1990).
- 20 15. Tanaka et al., Proc. Natl. Acad. Sci (USA) 91:3082-3086 (1994).
16. Barnstable et al., Cell 14:9-20 (1978).
17. Terry et al., Immunology 64:331-336 (1988).
18. Torimoto et al., Mol. Immunol. 29:183-192 (1992).
19. Lane et al., J. Prot. Chem. 10:151-160 (1991).
- 25 20. Malmqvist et al., Nature 361:186-187 (1993).
21. Nakanishi et al., Biological Mass Spectrometry 23:230-233 (1994).
22. Morrison et al., J. Exp. Med. 177:1135-1143 (1993).
23. Kyouden et al., J. Biochem. 111:770-777 (1992).
- 30 24. Hino et al., Clin. Chem. 22:1256-1261 (1976).

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25. Vanhoof et al., Eur. J. Clin. Chem. Clin. Biochem. 30:333-338 (1992).
26. Stancikova et al., Clin. Exp. Rheumatol. 10:381-385 (1992).
- 5 27. Hama et al., Clin. Chim. Acta. 113:217-221 (1981).
28. Chikuma et al., Biol. Chem. Hoppe-Seyler 371:325-330 (1990).
29. Fleischer et al., Immunology Today 15:180-184 (1994).
30. Schrader et al., J. Biol. Chem. 254:11964-11968 (1979).
- 10 31. Darmoul et al., Ann. Hum. Genet. 54:191-197 (1990).
32. Chobert et al., J. Biol. Chem. 265:2352-2357 (1990).
33. Wada et al., Proc. Natl. Acad. Sci. (USA) 89:197-201 (1992).
34. Scanlan et al., Proc. Natl. Acad. Sci. (USA) 91:5657-
15 5661 (1994).
35. Bernard et al., Biochemistry 33:15204-15214 (1994).
36. Ollis et al., Protein Engineering 5:197-211 (1992).
37. Hirschhorn et al., "Isozymes: Current Topics in
Biological and Medical Research" 4 (1980).
- 20 38. Niedzwicki et al., Am. J. Hematol. 37:152-155 (1991).
39. Ungerer et al., Clin. Chem. 38:1322-1326 (1992).
40. Matsuda et al., Clin. Infect. Dis. 16:260-264 (1993).

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Other Embodiments

The invention also includes fragments and analogs of the 175 kDa form of DPPIV/CD26. The term "analogs" refers to polypeptide fragments of the naturally occurring 175 kDa form of DPPIV/CD26 having conservative and/or non-conservative amino acid substitutions, having D-amino acids in place of some or all of the corresponding L-amino acids, or having non-peptide bonds in place of some of the peptide bonds of the naturally occurring form. Techniques for producing such analogs are well known in the art, and can be readily accomplished by those of ordinary skill. Preferably at least 85%, more preferably at least 95%, and most preferably at least 99%, of the amino acids in the analog are identical to the corresponding ones in the naturally occurring form. It is important that the substitutions do not eliminate the ability of the polypeptide fragment to interfere with the naturally occurring association between CD26 and CD45, or the ability of the compound to stimulate proliferation of lymphocytes. In some instances, the removal of peptide bonds from a polypeptide compound is a desirable goal because the presence of such bonds may leave the compound susceptible to attack by proteolytic enzymes. Additionally, such peptide bonds may affect the biological availability of the resulting therapeutic molecules. The removal of peptide bonds is part of a process referred to as "depeptidization." Depeptidization entails such modifications as replacement of the peptide bond (-CONH-) between two given amino acids with a spatially similar group such as -CH₂CH₂-, CH₂-O-, -CH=CH- or

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-CH₂S-, generally by incorporating a non-peptide mimetic of the dipeptide into the chemically synthesized analog of the invention.

Additional populations of polyclonal antibodies can be prepared as described herein by immunizing a suitable subject with 175 kDa DPPIV immunogen. For example, various host animals, including but not limited to rabbits, mice, hamsters, and rats can be immunized by injection with a preparation of the 175 kDa DPPIV or a specific peptide thereof. The preparation can further include an adjuvant, such as Freund's complete or incomplete adjuvant, or similar immunostimulatory agent. The anti-175 kDa DPPIV antibody titer in the immunized subject can be monitored over time by standard techniques, such as with an enzyme linked immunosorbent assay (ELISA) using immobilized 175 kDa DPPIV as an assay target. The antibody molecules directed against 175 kDa DPPIV can be isolated from the mammal and further purified by well known techniques, such as protein A chromatography, to obtain the IgG fraction.

Additional monoclonal antibodies with the desired specificity can be produced by obtaining antibody-producing cells from an immunized subject at an appropriate time after immunization, e.g., when the antibody titers are highest, and using the cells in any technique that provides for the production of antibody molecules by continuous cell lines in culture. These include, but are not limited to, the original techniques of Kohler and Milstein, *Nature*, 265:495-497 (1975); Brown et al., *J. Immunol.* 127:539-46 (1981); Brown

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et al., J. Biol. Chem. 255:4980-83 (1980); Yeh et al. PNAS 76:2927-31 (1976); and Yeh et al., Int. J. Cancer 29:269-75 (1985), the more recent EBV-hybridoma technique (Cole et al., Monoclonal Antibodies and Cancer Therapy, Alan R. Liss, Inc., 1985, pp.77-96) or trioma techniques. The technology for producing monoclonal antibody hybridomas is well known (see generally R. H. Kenneth, in Monoclonal Antibodies: A New Dimension In Biological Analyses, Plenum Publishing Corp., New York, New York (1980); E.A. lerner Yale [?] J. Biol. Med. 54:387-402 (1981); Gefter et al. Somatic Cell Genet. 3:231-36 (1977)).

Briefly, an immortal cell line (typically a myeloma) is fused to lymphocytes (typically splenocytes) from a mammal immunized with 175 kDa DPPIV as described above, generally in the presence of a fusion enhancing reagent, for example, polyethylene glycol. Typically, the immortal cell line (e.g., a myeloma cell line) is derived from the same mammalian species as the lymphocytes. For example, murine hybridomas can be made by fusing lymphocytes from a mouse immunized with an immunogenic preparation of the present invention with an immortalized mouse cell line. Preferred immortal cell lines are mouse myeloma cell lines that are sensitive to culture medium containing hypoxanthine, aminopterin and thymidine ("HAT" medium). Any of a number of myeloma cell lines may be used as a fusion partner according to standard techniques, e.g., the P3-NS1/1-Ag4-1, P3-x63-Ag8.653 or Sp2/O-Ag14 myeloma lines. These myeloma lines are available from the American Type Culture Collection

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(ATCC), Rockville, Md. The resulting cells, which include the fused hybridomas, are then allowed to grow in selective medium, e.g., HAT medium, and the surviving cells are grown in such medium using limiting dilution conditions. The cells are grown in a suitable container, e.g., microtiter wells, and the culture supernatants of the resulting hybridoma are screened to identify those hybridomas producing an antibody specific for the 175 kDa form of DPPIV.

As an alternative to preparing monoclonal antibody-secreting hybridomas, a monoclonal anti-175 kDa DPPIV antibody can be identified and isolated by screening a recombinant combinatorial immunoglobulin library (e.g., an antibody phage display library) with the 175 kDa form of DPPIV to identify and isolate antibodies or antibody fragments having antigenic specificity for this protein (e.g., see Sastry et al., PNAS 86:5728, 1989; Huse et al., Science 246:1275, 1989; and Orlandi et al., PNAS 86:3833, 1989). Briefly, after an animal is immunized with the 175 kDa form of DPPIV, the antibody repertoire of the resulting B-cell pool is cloned. Methods are generally known for directly obtaining the DNA sequence of the variable regions of a diverse population of immunoglobulin molecules by using a mixture of oligomer primers and PCR (e.g., see Larrick et al. Biotechniques 11:1520157, 1991; Larrick et al., Methods: Companion to Methods in Enzymology 2:106-110, 1991).

The V-gene library cloned from the immunization-derived antibody repertoire can be expressed by a population of display packages, preferably derived from filamentous phage,

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to form an antibody display library. Kits for generating and screening phage display libraries are commercially available (e.g., the Pharmacia Recombinant Phage Antibody System, Catalog No. 27-9400-01; and the Stratagene SurfZAPTM Phage Display Kit, Catalog No. 240612). Additionally, examples of methods and reagents particularly amenable for use in generating and screening antibody display libraries can be found in, for example, Ladner et al., U.S. Patent No. 5,223,409; Kang et al., International Publication No. WO 92/18619; Dower et al., International Publication No. WO 93/01288; McCafferty et al., International Publication No. WO 92/09690; Ladner et al., International Publication No. WO 90/02809; Fuchs et al., *Bio/Technology* 9:1370-72 (1991); Griffiths et al., *EMBO J* 12:725-34 (1993); Hawkins et al., *J. Mol. Biol.* 226:889-96 (1992); Clarkson et al., *Nature* 352:624-28 (1993); Gram et al., *PNAS* 89:3576-80 (1992); Garrad et al., *Bio/Technology* 9:1373-77 (1991); Hoogenboom et al., *Nucl. Acid Res.* 19:1433-37 (1991); Barbas et al., *PNAS* 88:7978-82 (1991); and McCafferty et al., *Nature* 348:552-54 (1990).

Once displayed on the surface of a display package (e.g., filamentous phage), the antibody library is screened with an antigen specific to the 175 kDa form of DPPIV to identify and isolate packages that express an antibody having specificity for this protein. Nucleic acid encoding the selected antibody can then be recovered from the display package (e.g., phage genome) and subcloned into other expression vectors via standard recombinant DNA techniques.

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Additionally, recombinant antibodies specific for the 175 kDa form of DPPIV, such as chimeric and humanized monoclonal antibodies, comprising both human and non-human portions, which can be made using standard recombinant DNA techniques, are within the scope of the invention. Such chimeric and humanized monoclonal antibodies can be produced by recombinant DNA techniques known in the art, for example, using methods described in Robinson et al., International Patent Publication PCT/US86/02269; Akira et al., European Patent Application 184,187; Taniguchi, European Patent Application 171,496; Morrison et al, European Patent Application 173,494; Neuberger et al., PCT Application WO 86/01533; Cabilly et al, U.S. Patent No. 4,816,567; Cabilly et al., European Patent Application 125,023; Better et al., *Science* 240:1041-43 (1988); Liu et al., *PNAS* 84:3439-43 (1987); Liu et al., *J. Immunol.* 139:3521-26 (1987); Sun et al., *PNAS* 84:214-18 (1987); Nishimura et al., *Canc. Res.* 47:999-1005 (1987); Wood et al., *Nature* 314:446-49 (1985); Shaw et al., *J. Natl. Cancer Inst.* 80:1553-59 (1988); Morrison, *Science* 229:1202-07 (1985); Oi et al, *BioTechniques* 4:214 (1986); Winter, U.S. Patent No. 5,225,539; Jones et al., *Nature* 321:552-25 (1986); Veroeyan et al., *Science* 239:1534 (1988); and Beidler et al., *J. Immunol.* 141:4053-60 (1988).

Screening procedures that can be used to screen hybridoma cells producing antibodies of the invention include, but are not limited to, enzyme-linked immunoadsorbent assays (ELISA) and immunoprecipitation. Many

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different ELISAs that can be used to screen for the antibodies can be envisioned by persons skilled in the art. These include, but are not limited to, formats comprising the purified or recombinantly produced 175 kDa form of DPPIV attached to a solid phase. Monoclonal antibodies that recognize an epitope on the 175 kDa form of DPPIV can then be tested for crossreactivity with the 105 kDa membrane or recombinant forms of CD26, to identify those which specifically recognize the 175 kDa form, or for ability to compete with other anti-175 kDa DPPIV monoclonal antibodies, e.g., the monoclonal antibodies identified herein.

Once the desired hybridoma has been selected and cloned, the resultant antibody can be produced in a number of ways. For example, the hybridoma can be cultured *in vitro* in a suitable medium for a suitable length of time, followed by the recovery of the desired antibody from the supernatant. The length of time and medium are known or can be readily determined. This *in vitro* technique produces essentially monospecific monoclonal antibody, essentially free from other species of anti-human immunoglobulin. To produce a large quantities of monoclonal antibody, the desired hybridoma can be injected into an animal, such as a mouse. Preferable, the mice are syngeneic or semi-syngeneic to the strain from which the monoclonal-antibody hybridomas were obtained. Injection of the hybridoma causes formation of antibody producing tumors after a suitable incubation time, which will result in a high concentration of the desired antibody (about 5-20 mg/ml) in the ascites of the host animal. Antibody molecules

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can be purified by known techniques, e.g., immunoabsorption or immunoaffinity chromatography, chromatographic methods such as high performance liquid chromatography or a combination thereof.

5 Monoclonal antibodies exhibiting the characteristics described herein can be of class IgG, subclass IgG₁, IgG_{2a}, IgG_{2b}, IgG₃, or of classes IgM, IgA, IgE. The differences among these classes or subclasses will not affect the selectivity of the reaction pattern of the antibody, but may
10 affect the further reaction of the antibody with other materials, such as for example complement or anti-mouse antibodies. The subject monoclonal antibodies described herein are IgM; however, it is contemplated that antibodies having the patterns of reactivity illustrated herein are
15 included within the subject invention regardless of the immunoglobulin class or subclass to which they belong. In general, antibodies of the IgG isotype are preferred. The desired isotype of an antibody can be selected by screening potential antibodies by an ELISA assay designed to select the
20 isotype of interest. For example, the solid phase of the assay can be coated with goat anti-mouse IgG Fc specific antibodies if it is desired to select for antibodies having the IgG isotype. It is possible that the immunized animal will not produce antibodies having the IgG isotype because
25 the immunogen is not recognized as sufficiently foreign. To overcome this obstacle, the structure of the immunogen may be slightly modified, e.g., by heat denaturization, and the

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animal reimmunized, with the produced antibodies being screened against the intact protein.

Alternatively, it is possible to switch the isotype of a given antibody. Many methods for accomplishing such a switch are known to those skilled in the art and include recombinant DNA techniques such as those described above to design an antibody having the desirable variable region fused to a constant region of the appropriate isotype. In addition, various isotype selection techniques are available. For example, the isotype switch can be done by repeatedly selecting for the isotype of interest using magnetic beads (e.g., super paramagnetic iron oxide particles; Biomag^(R) beads, Advanced Magnetix, Inc.) coated with a goat anti-mouse antibody preparation including all isotype classes. In switching the isotype from IgG₁ to IgG_{2a}, for instance, the IgG_{2a} binding sites on the coated magnetic beads are first blocked with an irrelevant antibody of the IgG_{2a} isotype. All cells producing antibodies of differing isotypes will then be bound by the beads and removed magnetically, resulting in an enrichment of cells producing the IgG_{2a} isotype. These cells can then be cloned by limiting dilution, and using commercially available anti-isotypic reagents in an ELISA assay, the IgG_{2a} producing clones can be identified. A method which facilitates selecting for the isotype of interest is exemplified, for example, in PCT International Publication No. WO 90/06758.

Moreover, while the specific examples described herein are from a rabbit or mouse, this is not meant to be a

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limitation. The antibodies of the invention having the desired specificity, whether from a murine source, other mammalian source including rabbit, rat human, or other sources, or combinations thereof, are included within the scope of this invention.

The antibodies can be used for the detection and/or enumeration by indirect staining of activated T lymphocytes in normal individuals or in disease states, for example, by fluorescence microscopy, flow cytometry, immunoperoxidase, or other indirect methodologies.

Also included within the scope of the present invention are antibody fragments and derivatives which comprise at least the functional portion of the antigen binding domain of an antibody that binds specifically to the 175 kDa form of DPPIV. Antibody fragments that contain the binding domain of the molecule can be generated by known techniques. For example, such fragments include, but are not limited to: the $F(ab')_2$ fragment which can be produced by pepsin digestion of the antibody molecule; the Fab' fragments which can be generated by reducing the disulfide bridges of the $F(ab')_2$ fragment, and the Fab fragments which can be generated by treating the antibody molecule with papain and a reducing agent. See, e.g., National Institutes of Health, 1 Current Protocols In Immunology, Coligan et al., ed. §§ 2.8, 2.10 (Wiley Interscience, 1991). Antibody fragments also include Fv fragments, i.e., antibody products in which there are no constant region amino acid residues. Such fragments can be

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produced, for example, as described in U.S. Patent No. 4,642,334.

Chimeric antibody derivatives are also contemplated to be within the scope of the present invention. Chimeric antibody molecules can include, for example, the antigen binding domain from an antibody of a mouse, rat, or other species, with human constant regions. Different approaches for making chimeric antibodies have been described and can be used to make chimeric antibodies containing the immunoglobulin variable region which recognizes the 175 kDa form of CD26. See, for example, Morrison, et al, Proc. Natl. Acad. Sci. USA 81:6851 (1985); Takeda et al. Nature 314:452 (1985), Cabilly et al., U.S. Patent No. 4,816,567; Boss et al, U.S. Patent No. 4,816,397.

While the present invention has been described in conjunction with a preferred embodiment, one of ordinary skill, after reading the foregoing specification, will be able to effect various changes, substitutions of equivalents, and other alterations to the compositions and methods set forth herein. It is therefore intended that the protection granted by Letters Patent hereon be limited only by the definitions contained in the appended claims and equivalents thereof.

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CLAIMS

What is claimed is:

- 1 1. A substantially purified glycoprotein having a molecular
2 weight of approximately 175 kDa, of which approximately 130
3 kDa is the unglycosylated moiety, wherein said glycoprotein
4 exhibits functional dipeptidylpeptidase IV (DPPIV) activity
5 but not Adenosine Deaminase Type-1 (ADA-1) binding activity.

- 1 2. A substantially purified protein having a molecular
2 weight of approximately 130 kDa, said protein being the
3 unglycosylated moiety of the glycoprotein of claim 1.

- 1 3. The glycoprotein of claim 1, said glycoprotein being
2 complexed as part of a trimer.

- 1 4. The glycoprotein of claim 1, said glycoprotein having
2 N-linked glycosylation but not O-linked glycosylation.

- 1 5. The glycoprotein of claim 1, wherein said glycoprotein
2 is obtainable from normal human plasma or serum.

- 1 6. A therapeutic composition comprising the glycoprotein
2 of claim 1 in a pharmaceutically acceptable carrier
3 substance.

- 1 7. A method for enhancing the immune response of an
2 immunosuppressed patient comprising

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3 administering to said patient a therapeutically
4 effective amount of the therapeutic composition of claim 6.

1 8. An antibody that binds specifically to the 175 kDa form
2 of DPPIV/CD26 but not to the 105 kDa form of DPPIV/CD26.

1 9. The antibody of claim 8, wherein said antibody is a
2 polyclonal antibody.

1 10. The antibody of claim 8, wherein said antibody is a
2 monoclonal antibody.

1 11. The antibody of claim 8, wherein said antibody is a
2 human antibody.

1 12. A method for determining the extent of T cell activation
2 in a patient comprising
3 detecting the concentration of the 175 kDa form of
4 DPPIV/CD26 in plasma or serum from said patient.

1 13. The method of claim 12 wherein said detecting step
2 comprises
3 providing a biological sample from a patient,
4 contacting said sample with the antibody of claim 8,
5 and detecting the extent of binding of said antibody to
6 said sample.

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1 14. A kit for determining the extent of T cell activation
2 in a patient comprising the antibody of claim 8.

1 15. The kit of claim 14 further comprising means for
2 determining the amount of protein in a biological sample and
3 means for comparing the extent of binding of said antibody
4 to said sample with a standard.

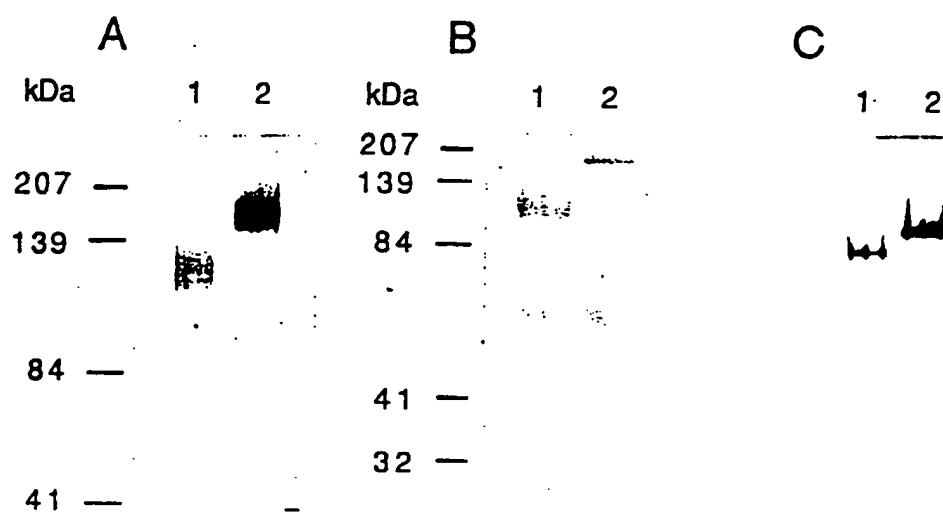


Fig. 1

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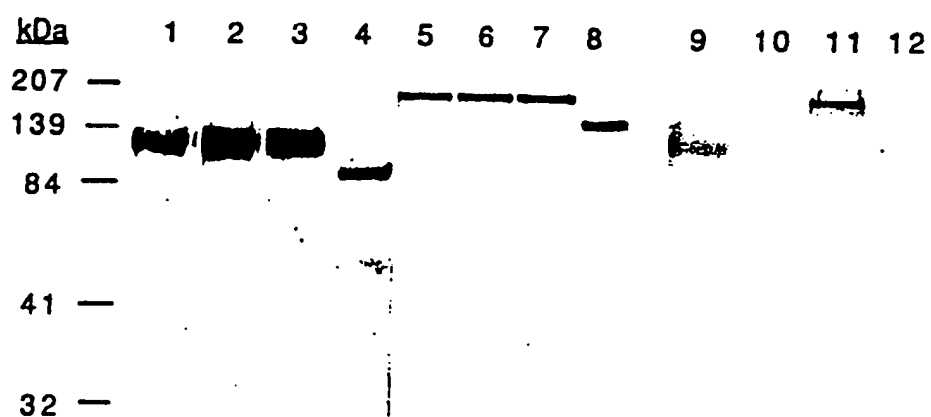


Fig.2.

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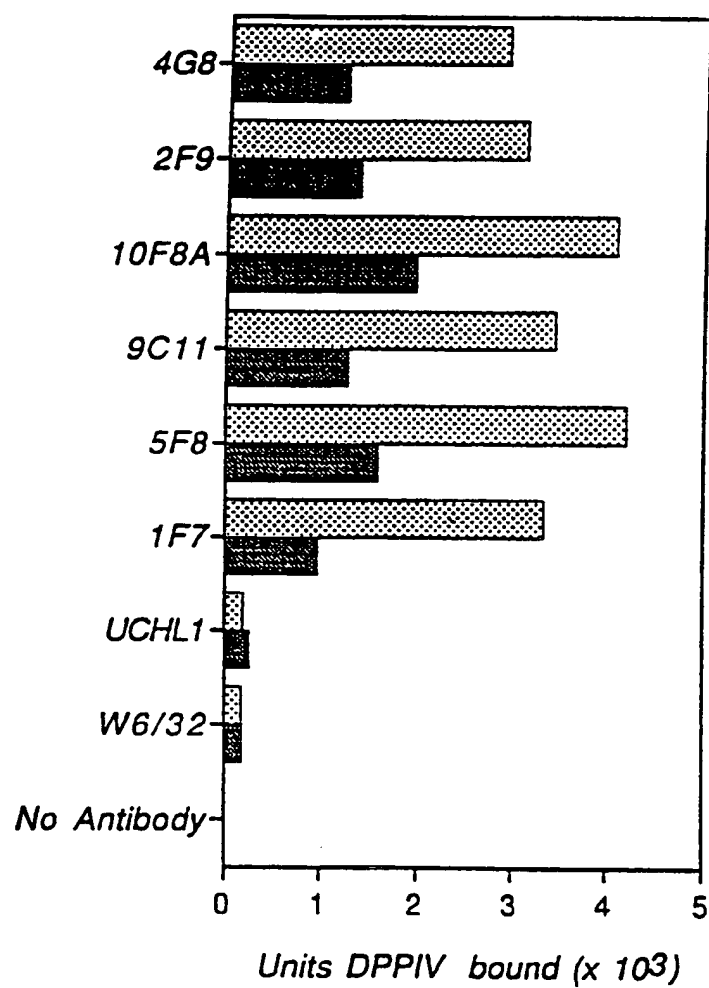


Fig. 3.

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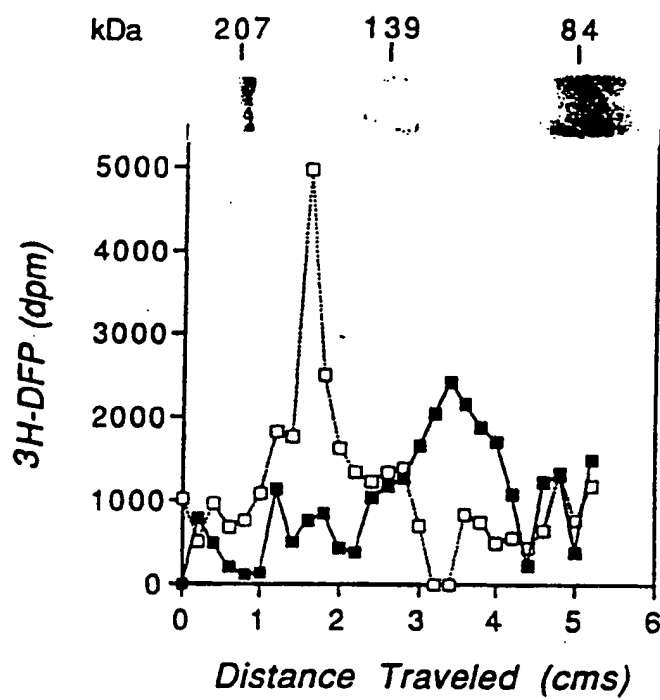


Fig. 4.

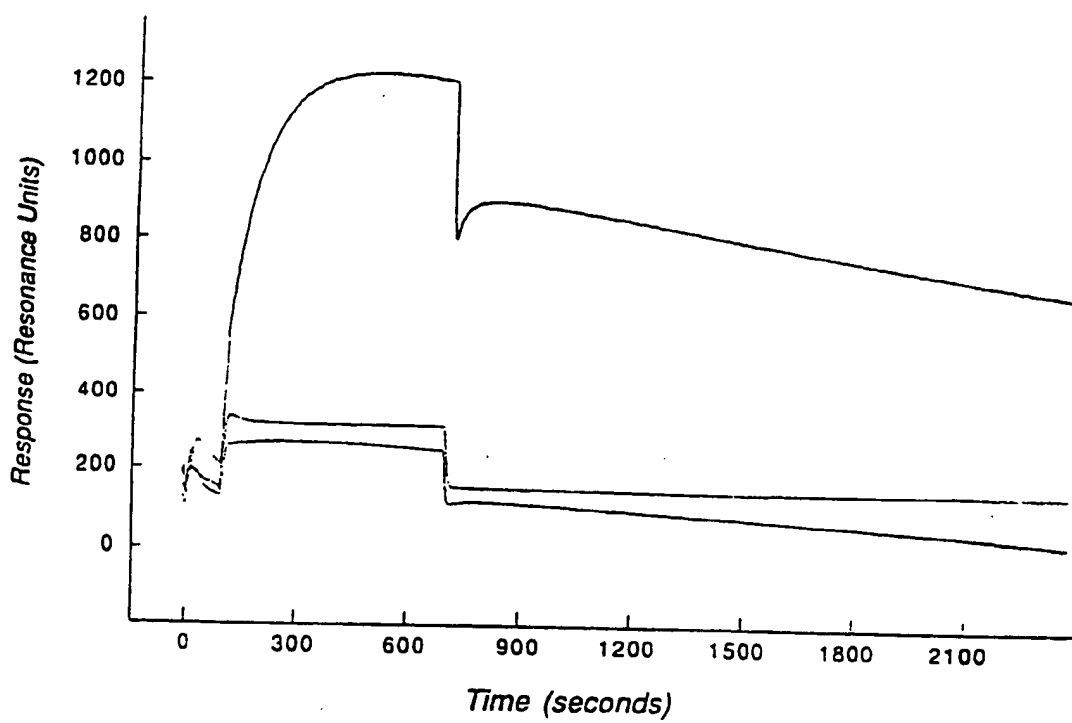


Fig. 5A

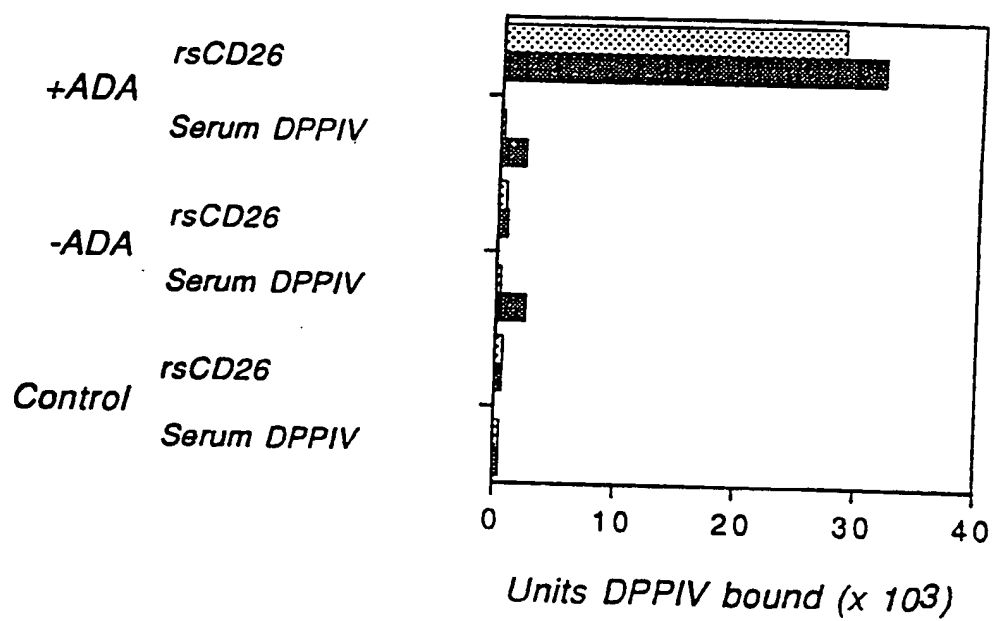


Fig. 5B

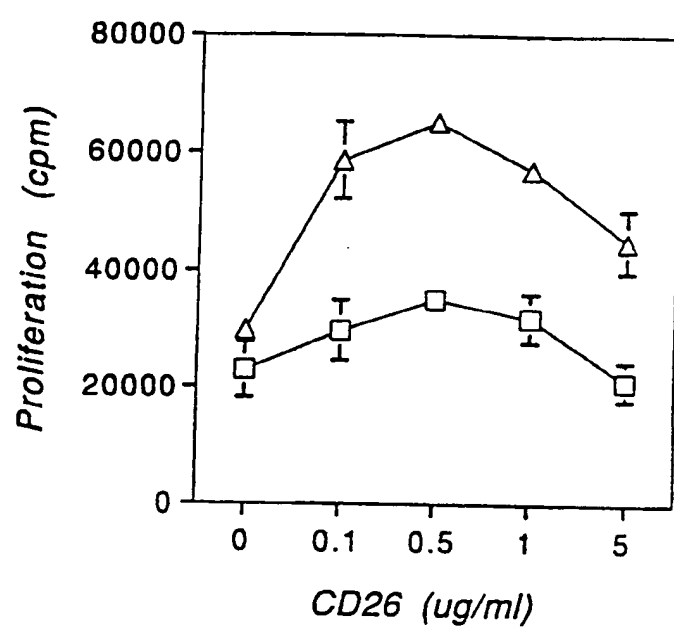


Fig. 6.

PHA-activated T cells release soluble DPPIV/CD26. molecular weight of released material is 175 kDa, and appears to be identical with serum soluble CD26/DPPIV.

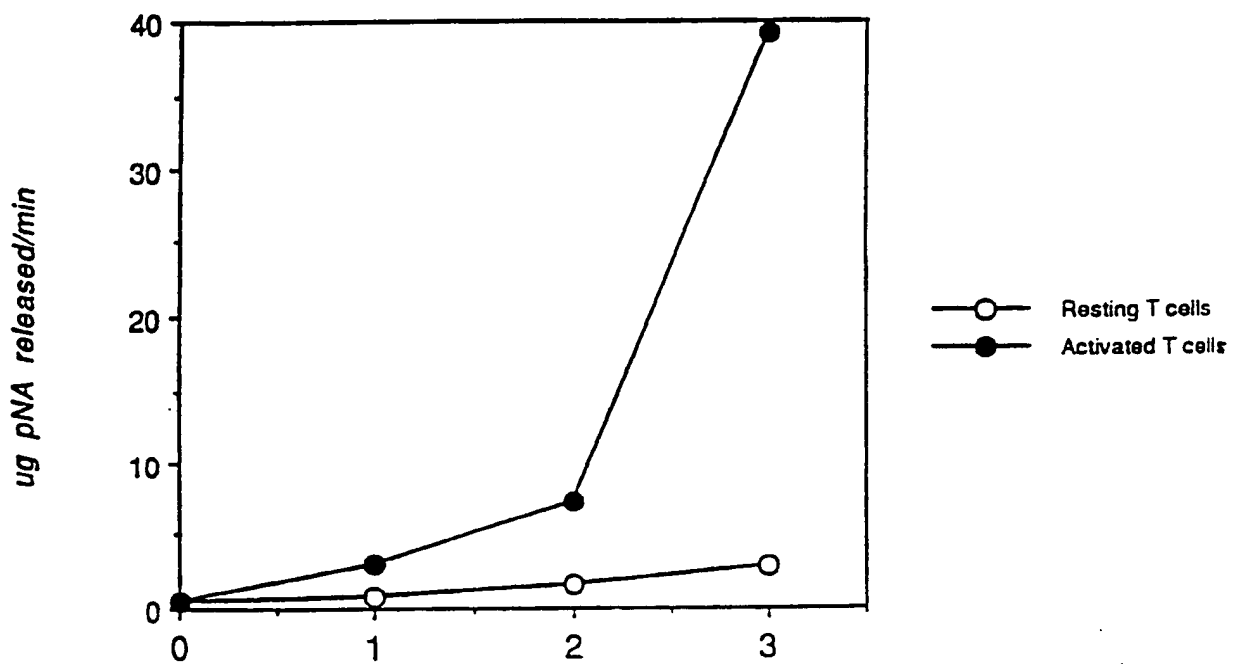
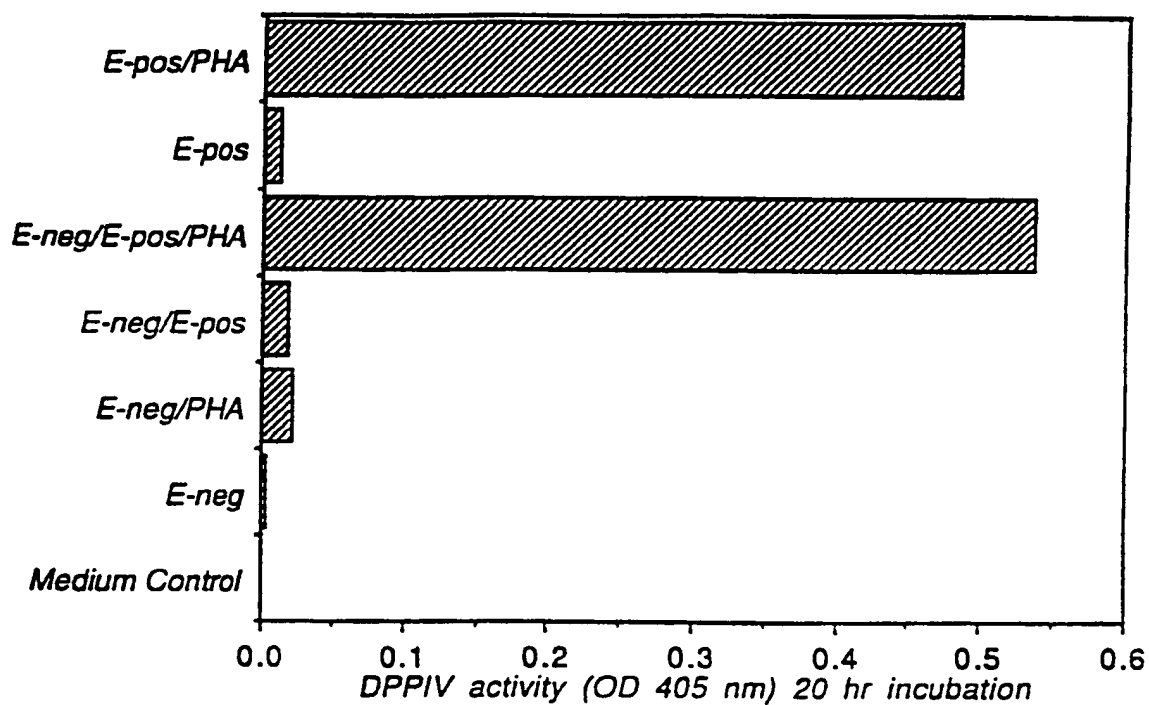


Fig. 7

Only activated T cells release CD26/DPPIV - it is not released by E-negative cells - monocytes/macrophages/B cells.



DPPIV activity of supernatants of indicated cells - 4 day incubation

Fig. 8

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Recombinant soluble CD26, purified serum CD26/DPPIV, and purified T cell-secreted soluble CD26/DPPIV can all co-stimulate the response of normal T cells to the recall antigen, tetanus toxoid.

Response to Tet. Tox - Effect of sCD26, pCD26, iCD26

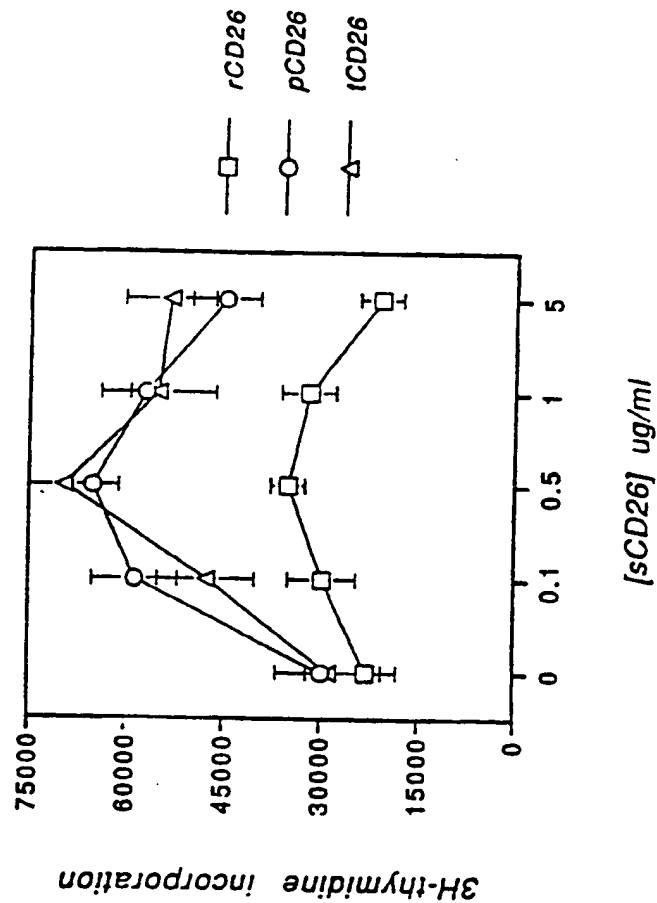


Fig. 9

INTERNATIONAL SEARCH REPORT

International Application No
PC1/US 96/08370

A. CLASSIFICATION OF SUBJECT MATTER
IPC 6 C12N9/48 C07K16/18 C07K14/00 A61K39/00 G01N33/53

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 6 C12N A61K C07K G01N

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
P,X	JOURNAL OF IMMUNOLOGY, vol. 156, no. 5, 1996, pages 1714-1721, XP002013092 J.S. DUKE-COHAN ET AL.: "Serum high molecular weight DP1V (CD26) is similar to a novel antigen DPPT-L released from activated T cells" *see the whole article*	1-15
P,X	JOURNAL OF BIOLOGICAL CHEMISTRY, vol. 270, no. 23, 1995, pages 14107-14114, XP002013093 J.S. DUKE-COHAN ET AL.: "A novel form of DPPIV found in human serum" *see the whole article*	1-15

☐ Further documents are listed in the continuation of box C.

☐ Patent family members are listed in annex.

* Special categories of cited documents:

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Date of the actual completion of the international search

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23. 10. 96

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